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Transcriptomic Response of *Lactococcus lactis* in Mixed Culture with *Staphylococcus aureus*▽†

Sébastien Nouaille,[1,2,3]* Sergine Even,[4,5] Cathy Charlier,[4,5] Yves Le Loir,[4,5] Muriel Cocaign-Bousquet,[1,2,3] and Pascal Loubière[1,2,3]

Université de Toulouse, INSA, UPS, INP, LISBP, 135 Avenue de Rangueil, F-31077 Toulouse, France[1]; INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France[2]; CNRS, UMR5504, F-31400 Toulouse, France[3]; INRA, UMR1253 STLO, 85 Rue de Saint Brieuc, 35042 Rennes Cedex, France[4]; and Agrocampus Ouest, UMR1253 STLO, 85 Rue de Saint Brieuc, 35042 Rennes Cedex, France[5]

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The mechanisms of interaction between *Lactococcus lactis* and the food pathogen *Staphylococcus aureus* are of crucial importance, as one major role of lactic acid bacteria (LAB) in fermented foods is to inhibit undesirable and pathogenic flora. It was never questioned if the presence of a pathogen can actively modify the gene expression patterns of LAB in a shared environment. In this study, transcriptome and biochemical analyses were combined to assess the dynamic response of *L. lactis* in a mixed culture with *S. aureus*. The presence of *S. aureus* hardly affected the growth of *L. lactis* but dramatically modified its gene expression profile. The main effect was related to earlier carbon limitation and a concomitantly lower growth rate in the mixed culture due to the consumption of glucose by both species. More specific responses involved diverse cellular functions. Genes associated with amino acid metabolism, ion transport, oxygen response, menaquinone metabolism, and cell surface and phage expression were differentially expressed in the mixed culture. This study led to new insights into possible mechanisms of interaction between *L. lactis* and *S. aureus*. Moreover, new and unexpected effects of *L. lactis* on the virulence of *S. aureus* were discovered, as described elsewhere (S. Even, C. Charlier, S. Nouaille, N. L. Ben Zakour, M. Cretenet, F. J. Cousin, M. Gautier, M. Cocaign-Bousquet, P. Loubière, and Y. Le Loir, Appl. Environ. Microbiol. 75:4459–4472, 2009).

Lactococcus lactis, the model organism of the lactic acid bacteria (LAB), is used extensively in numerous food fermentation processes, particularly in cheese production. While its main technological function is medium acidification, another important role is the inhibition of the growth of food pathogens or undesirable microorganisms, ensuring the safety and quality of the fermented products. *Staphylococcus aureus* is a major human pathogen causing a variety of infections, ranging from minor skin and wound infections to life-threatening diseases (26). In addition to its natural ecological niches, which are the nasal cavity and the skin, *S. aureus* can be a foodstuff-contaminating agent, leading to food poisoning through the production of staphylococcal enterotoxins (24). As *S. aureus* shares the same ecosystem with LAB, an increasing number of studies have focused on the inhibitory effects of LAB on *S. aureus* growth. The major parameters involved in bacterial growth inhibition reported so far are the pH decrease by the production of organic acid, nutrient competition, and hydrogen peroxide and antibiotic production (6). Most of the studies have been restricted to quantifying the potential of LAB for inhibiting pathogen growth, and little is known about the mechanisms leading to growth inhibition and whether other parameters may be associated with microbial interaction. The behavior of *L. lactis* in the presence of food pathogens, particularly *S. aureus*, has never been clearly analyzed. It is not known to date whether *L. lactis* can sense the partner organism and can actively modify its gene expression pattern to fight against the pathogen or just passively respond to the bacterial partner. Global approaches appear to be powerful tools to investigate multifactorial interactions. Microarrays have been employed largely to identify cellular responses, in both *L. lactis* and *S. aureus*, to different stress or environmental modifications (32, 37, 39, 41, 46), but a transcriptomic approach with mixed cultures of these two microorganisms has never been carried out.

In this study, we analyzed the transcriptomic response of a dairy strain of *L. lactis* in a mixed culture with *S. aureus*. Cultures were carried out in fermentors in a chemically defined medium (CDM) and at a constant pH to avoid acidification effects. To mimic the cheese-making industrial processes, no oxygen control was applied. The main response of *L. lactis* in the mixed culture could be attributed primarily to the earlier modification of the environmental composition imposed by the staphylococcal growth, but more specific cellular functions were also found to be modified, highlighting the interest of the transcriptome for the analysis of microbial interactions.

* Corresponding author. Mailing address: INSA-LISBP, 135 avenue de Rangueil, 31077 Toulouse Cedex 4, France. Phone: 33 (0) 5 61 55 94 38. Fax: 33 (0) 5 61 55 94 00. E-mail: sebastien.nouaille@insa-toulouse.fr.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* subsp. *lactis* biovar diacetylactis LD61 (kindly provided by R. Perrin, Soredab, La Boissière Ecole, France) and *S. aureus* MW2 (control cultures) and mixed cultures containing the two microorganisms were grown on CDM (22) at 30°C in 2-liter fermen-

TABLE 1. Comparison of microarray and quantitative PCR (qPCR) data

Gene name	Sequences of primer pairs for qPCR	Ratio[a] of expression in mixed culture to that in pure culture as determined by:			
		Transcriptome analysis		qPCR	
		7.8 h	9 h	7.8 h	9 h
citD	5′-CGAGGCTACGGTGCGTCAAG-3′/5′-TTCCACTACCGCAATCGCTCTG-3′	2.2	2.0	2.4	4.6
serS	5′-GCCATTGATGCTGAACTTGCTG-3′/5′-ACCAACACGACGAACCTCAAC-3′	3.1	3.6	2.7	4.4
menX	5′-TCGTCCGCCCATTGAATAGCC-3′/5′-GGTCACGGTTCATCAGCAAAGC-3′	1.9		1.1	
msmK	5′-GCTTGACCGTAAACCTGCTGAC-3′/5′-ACTTTGGCATCACGGACAATCG-3′		2.8		13.2
mtsA	5′-ACCTCAACTCCAGTGCTTACAG-3′/5′-ACTTGGAAACAGGTGGAAATGC-3′		2.5		11.3
rbsK	5′-GGCTTGGTTCGCTCCTTTTCC-3′/5′-GAGTGCCGAATGCTGGAGAAAC-3′		5.7		7.3
yahB	5′-CTGAGCAGGCAGAAGAAGCG-3′/5′-CTGGAATAGCTGGTGCAGGAAC-3′		1.9		1.7
yobA	5′-AACTCCGCTTCAGACCATTGC-3′/5′-GTTCCTGTCGATGGTTCTGACC-3′		4.4		7.3
ytgH	5′-GAGCATCATCGCCCATGTCATC-3′/5′-GCGCAGCTCAAACAACAGGG-3′		2.6		3.2
rcfA	5′-CGGACAACTTTATGGTCAGGCC-3′/5′-GCTTGGTGCTCAGCAGCTAG-3′				

[a] Expression ratios in each type of culture were calculated relative to expression at the 5-h time point.

tors (Setric Génie Industriel, Toulouse, France) with an agitation speed of 200 rpm, and the pH was maintained at 6.6 by the automatic addition of KOH (10 N) to avoid the acidification of the medium by lactic acid produced mainly by *L. lactis*. Fermentations were carried out under oxygen-limiting conditions, with air in the gaseous phase but without air bubbling. Cultures were inoculated from precultures grown in tubes containing CDM at 30°C. Predetermined volumes of each exponentially growing preculture were added to mixed and control cultures to obtain initial cell concentrations of 10^6 CFU ml^{-1} for both *S. aureus* and *L. lactis*. For each condition (pure *L. lactis* culture, pure *S. aureus* culture, and mixed culture), three independent biological replicates were established.

Analytical methods. Bacterial growth was estimated by spectrophotometric measurements at 580 nm with a Hitachi U1100 spectrophotometer (1 U of absorbance is equivalent to 0.3 g $liter^{-1}$ for both *L. lactis* and *S. aureus*) and the determination of numbers of CFU by a micromethod as described previously (1). The populations of *L. lactis* and total flora were determined after growth on M17 (Difco) (44) agar plates supplemented with 0.5% glucose and incubated for 24 h at 30°C. The population of *S. aureus* was determined after growth on tryptic soy broth (AES, Combourg, France) agar plates supplemented with 6.5% NaCl and incubated for 24 h at 37°C. Glucose, lactate, acetate, citrate, pyruvate, formate, and ethanol concentrations in culture supernatants were measured by high-performance liquid chromatography with a 1200 series system (Agilent Technologies, Waldbronn, Germany) using an HPX87H$^+$ Bio-Rad column and the following conditions: a temperature of 48°C, H_2SO_4 (5 mM) as the eluent at a flow rate of 0.5 ml min^{-1}, and dual detection by refractometer and UV analyses. Amino acid concentrations in culture supernatants were measured via the AminoQuant HP1090 system (Agilent Technologies, Waldbronn, Germany). Proteins in the samples were precipitated by adding 4 volumes of methanol to 1 volume of the sample and incubating the mixture overnight on ice. The mixture was then centrifuged, and the supernatant was kept for amino acid analysis. The amino acids were automatically derived with *ortho*-phthalic aldehyde and 9-fluorenylmethyl chloroformate. The derivatives were separated on a Hypersil AA octyldecyl silane column (Agilent Technologies) at 40°C by using a linear gradient of acetate buffer (pH 7.2) with triethylamine (0.018%), tetrahydrofuran (0.3%), and acetonitrile. A diode array detector was used at 338 nm for *ortho*-phthalic aldehyde derivatives and at 262 nm for 9-fluorenylmethyl chloroformate derivatives.

Transcriptomic analysis. Cells were collected at exponential growth phase, late exponential growth phase, early stationary phase, and stationary phase, at 5.0, 7.8, 9.0, and 11.1 h postinoculation, respectively, and immediately frozen in liquid nitrogen. After defrosting of the cells on ice, a volume corresponding to 6 mg (dry weight) of cells was centrifuged. Cells were broken at 4°C with a FastPrep-24 instrument (MP Biomedicals, Illkirch, France) by three cycles of 1 min interspaced with 2-min cooling periods. Total RNAs were extracted using an RNeasy midi kit (Qiagen) as described previously (39). RNAs were quantified at 260 nm, and RNA quality was controlled on an electrophoresis agarose gel under denaturing conditions. For cDNA synthesis, 20 μg of total RNA was retrotranscribed as described previously (39). *S. aureus* MW2 genomic DNA was extracted essentially as described previously (2) and digested with Sau3A (1 U/93 μg of DNA for 1 h at 37°C). Gene expression was measured using nylon arrays containing the PCR fragments (Eurogentec) of 1,948 genes of *L. lactis* IL1403 (4). Membrane spotting and analytical support were provided by the biochip platform of Genopole (Toulouse, France). Membrane preparation and hybridization conditions were similar to those described previously (39), with the sole modification of the addition of 40 μg of Sau3A-digested MW2 genomic DNA, denatured at 95°C for 10 min, to the hybridization buffer. Membranes were exposed to a phosphorimager screen for 3 days and scanned with a phosphofluoroimager (Storm 860; Molecular Dynamics).

Data analyses and statistical treatment. Hybridization signals were quantified, assigned to gene names, and statistically evaluated with the Bioplot software (developed by S. Sokol of Plateforme Génomique, Toulouse [http://biopuce.insa-toulouse.fr]). Local background was removed, and signals were normalized according to the mean intensity of the membrane. Ratio calculations and statistical analyses were restricted to genes whose detected signal was above the cutoff level, defined as the mean of the signals detected for the 180 empty spots present on the membrane plus 2 standard deviations. For each culture, the expression ratios were calculated using the exponential phase (5 h) as the reference. Student tests were performed, and genes with false-discovery rates (FDR) of <3% were selected. These genes have *P* values under the threshold of 0.082, 0.023, 0.045, 0.017, 0.021, and 0.016 in pure (7.8 h), mixed (7.8 h), pure (9 h), mixed (9 h), pure (11.1 h), and mixed (11.1 h) cultures, respectively. To find cross-hybridizing genes, we used cDNA from the *S. aureus* pure culture. Genes whose signal was above the cutoff were designated cross-hybridizing genes. Genes exhibiting significant variations in signal intensities at the studied time points were selected by using a Student test. The distribution of the *P* values for this limited number of genes was not Gaussian, and it was not possible to calculate the FDR. A threshold *P* value of 0.05 was chosen for gene selection in this case.

Real-time PCR. Samples of 10 μg of total mRNA were retrotranscribed exactly as described previously (27). Primers for real-time PCR (Table 1) were designed with Bio-Rad Beacon Designer software to have lengths of 20 to 24 bases, GC contents of more than 50%, and melting temperatures of about 60°C and to amplify PCR products of 90 to 150 bases long. The specificities of the primers for the genes of interest were controlled by using the *L. lactis* IL1403 genome with Vector NTI software (Invitrogen). Similarly, the absence of primer specificity for the *S. aureus* MW2 genome was controlled and confirmed by the lack of amplification of MW2 genomic DNA by PCR. Real-time PCR was carried out on a MyIQ unit with Sybr green supermix as described previously (27). The threshold was determined with a baseline at 125 relative fluorescence units above the background level. Three dilutions of the cDNA were performed to determine the PCR efficiency (ranging from 85 to 116%). The *rcfA* gene was chosen as an internal normalization control, as *L. lactis* pure-culture transcriptomic data showed that its expression remained constant throughout growth. The Pfaffl analysis method (34) was used to calculate the change in transcript levels between pure and mixed cultures. Each selected gene in three culture replicates was analyzed. For direct comparison with transcriptomic data, quantitative reverse transcription-PCR results were expressed as differences (*n*-fold) in transcript concentrations between the mixed and pure cultures, with correction by using the *rcfA* normalization ratio. Ratios of gene expression levels in mixed and pure cultures analyzed by quantitative reverse transcription-PCR (Table 1) agreed with differences observed by the transcriptomic approach.

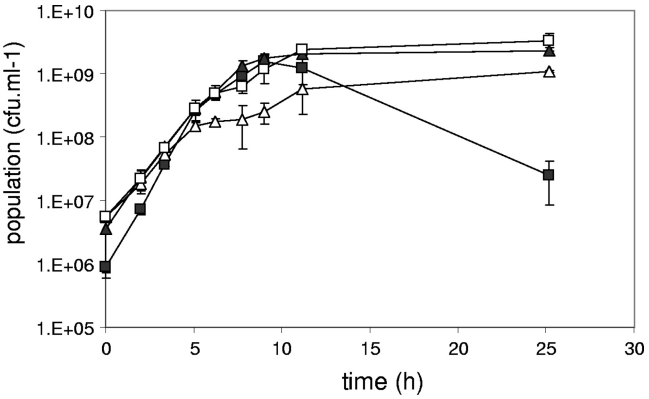

FIG. 1. Kinetics of growth of *L. lactis* LD61 and *S. aureus* MW2 in pure and mixed cultures on CDM medium at 30°C and a constant pH. Growth curves for *L. lactis* LD61 in the pure culture (■), the total population in the mixed culture (▲), *S. aureus* MW2 in the pure culture (□), and *S. aureus* MW2 in the mixed culture (△) are shown.

RESULTS AND DISCUSSION

***L. lactis* hardly affected the growth of *S. aureus* on CDM at a constant pH, and vice versa.** The kinetics of growth of pure and mixed cultures of *L. lactis* LD61 and *S. aureus* MW2 are presented in Fig. 1. The growth of *L. lactis* in the pure culture was exponential for the first 6 h, with a maximal rate of 1.05 h^{-1}, which was followed by a growth deceleration phase to 8.2 h, and the culture reached a final population of 2×10^9 CFU ml^{-1} at 9 h (Fig. 1). After 9 h, the population decreased to 3×10^7 CFU ml^{-1} at 25 h (Fig. 1), which was associated with cell lysis (the optical density dropped from 6 to 2.5 between 9 and 25 h [data not shown]). In the mixed culture, a decrease in the *L. lactis* biomass was not detectable based on the total population, due to the growing *S. aureus* subpopulation. However, lysis occurred in the mixed culture also and was even more pronounced than that in the pure culture, as confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with the release of cellular protein into the culture supernatant (data not shown).

The growth of *S. aureus* in the pure culture was exponential, with a maximal rate of 0.76 h^{-1} for the first 6 h, until the population reached 5×10^8 CFU ml^{-1}. From 6 h to the entry into the stationary phase (at 11 h), a period of reduced growth ($\mu = 0.32\ h^{-1}$) occurred (Fig. 1). This biphasic growth pattern may be related to nutrient exhaustion that led to metabolic adaptation (the induction of new metabolic pathways, transporters, and de novo biosynthesis pathways). The final *S. aureus* population after 25 h of culture was 3×10^9 CFU ml^{-1}. Similar growth of *S. aureus* in the mixed culture with *L. lactis*, but with a lag phase between the two growth phases, was observed. Following an exponential phase of growth ($\mu = 0.70\ h^{-1}$) of 5 h, similar to that in the pure culture, growth stopped for 3 h at an *S. aureus* population of 2×10^8 CFU ml^{-1}. A second phase of growth ($\mu = 0.32\ h^{-1}$) started after 8 h of culture. The entry into the stationary phase occurred earlier than that in the pure culture, an effect that may have been related to the anticipated exhaustion of nutrients due to competition with *L. lactis*. At 25 h, the *S. aureus* population in the mixed culture reached 10^9 CFU ml^{-1}, which was only threefold lower than that in the pure culture. General growth profiles of *S. aureus* in pure and mixed cultures were similar but showed a delay in the mixed culture due to growth arrest between 5 and 8 h. *S. aureus* did not seem to take advantage of nutrients released by *L. lactis* cell lysis, as growth rates in the slow-growth phases of the pure and mixed cultures were similar.

Kinetic profiles of the substrate (glucose) and fermentation products were determined. The *L. lactis* pure culture presented a classical profile of glucose consumption concomitant with lactate production, with a global lactate yield ($Y_{lactate/glucose}$) of 1.69 mol mol $glucose^{-1}$ (Fig. 2A). Small amounts of formate ($Y_{formate/glucose} = 0.05$ mol mol^{-1}) and acetate ($Y_{acetate/glucose} = 0.08$ mol mol^{-1}) were also produced after 6 h (Fig. 2B and C). The complete glucose exhaustion occurring at 9 h correlated with the entry into the stationary phase. *S. aureus* in the pure culture consumed glucose at a lower rate. Less than half of the glucose was consumed within the first 12 h, and it was finally exhausted at 25 h (Fig. 2A). *S. aureus* produced lactate as the main fermentation product ($Y_{lactate/glucose} = 1.57$ mol mol^{-1}). After 4 h of growth, formate ($Y_{formate/glucose} = 0.07$ mol mol^{-1}) and acetate ($Y_{acetate/glucose} = 0.05$ mol mol^{-1}) were produced in addition to lactate. Finally, after 7 h, ethanol was also produced at significant levels ($Y_{ethanol/glucose} = 0.22$ mol mol^{-1}) and reached a final concentration of 10.1 mM (Fig. 2B and C).

For the mixed culture, the glucose consumption profile was similar to that for the *L. lactis* pure culture, but glucose exhaustion occurred 1 h earlier in the mixed culture due to the concomitant consumption of glucose by both species. Similarly, the lactate concentration in the mixed culture was increased compared to its concentration in the *L. lactis* pure culture, but lactate production stopped earlier, when glucose was exhausted. The lactate concentration in the mixed culture then decreased, suggesting the consumption of lactate by *S. aureus*, as the lactate concentration in the *L. lactis* pure culture remained stable. The formate concentration in the mixed culture increased more rapidly than that in the *S. aureus* pure culture and reached 10 mM at the time of glucose exhaustion and 20 mM at 25 h. In contrast, ethanol production was delayed 1 h in the mixed culture compared to that in the *S. aureus* pure culture and started only after glucose exhaustion, with the ethanol concentration reaching a maximal level of 4.4 mM at 25 h, compared to 10.1 mM in the *S. aureus* pure culture. Finally, the fermented products excreted in the mixed culture were lactate, formate, acetate, and ethanol, produced with respective yields of 1.26, 0.41, 0.41, and 0.09 mol mol $glucose^{-1}$. However, this overall balance should be divided into two distinct phases, the first one occurring at the expense of glucose consumed by both species and leading to the production of lactate, formate, and acetate and the second phase corresponding to lactate consumption by *S. aureus* and the production of formate, acetate, and ethanol, with global yields of 1.18, 1.25, and 0.29 mol mol $lactate^{-1}$, respectively. The longer *S. aureus* lag phase observed in the mixed culture may represent the time required for metabolic adaptation to lactate consumption.

As expected, *L. lactis* LD61 demonstrated classical homolactic metabolism, with lactate as the main fermentation product. However, in the three types of cultures, formate, acetate,

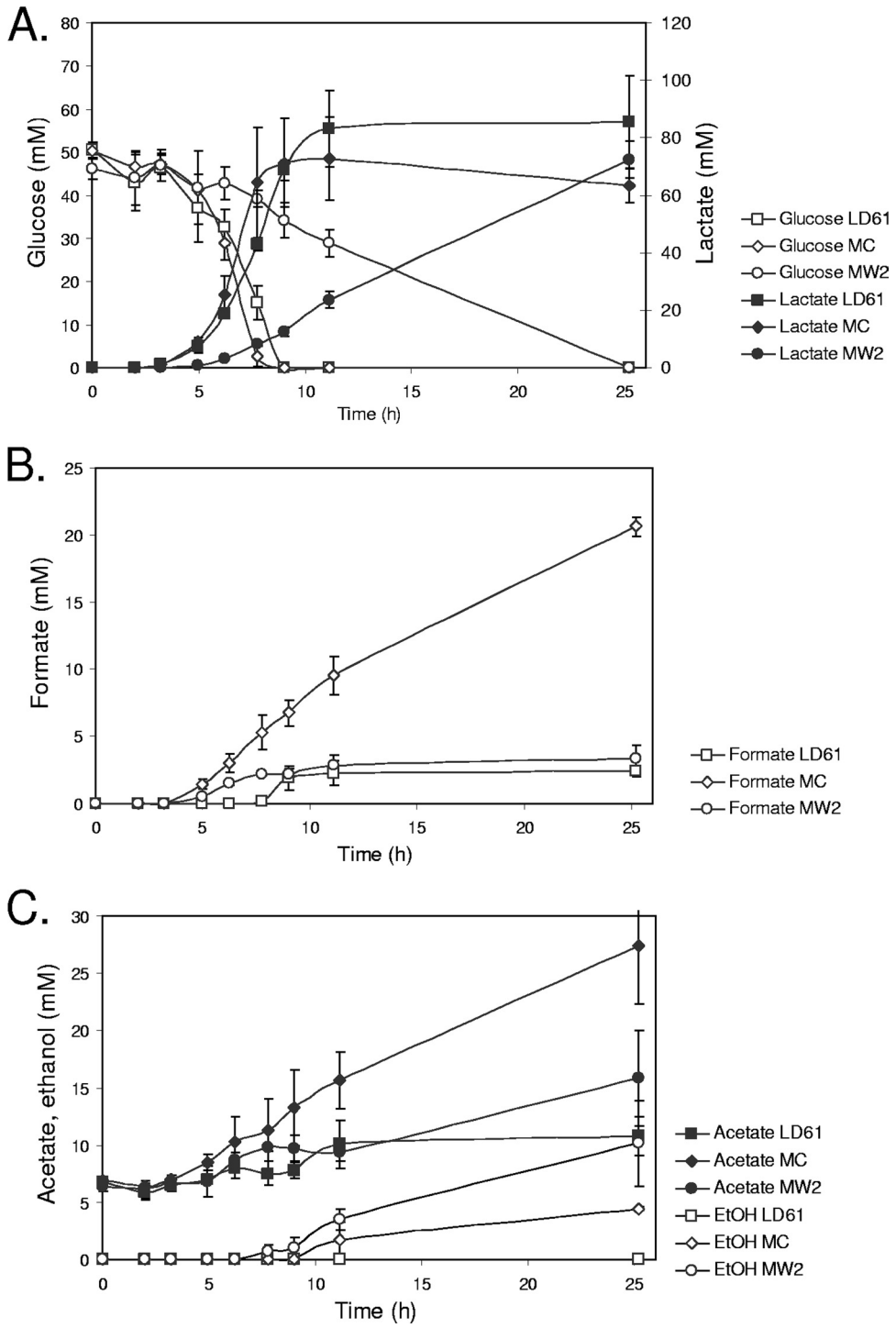

FIG. 2. (A) Glucose consumption by *L. lactis* LD61 in the pure culture (glucose LD61) and the mixed culture (glucose MC) and by *S. aureus* MW2 in the pure culture (glucose MW2) and lactate production by *L. lactis* LD61 in the pure culture (lactate LD1) and the mixed culture (lactate MC) and by *S. aureus* MW2 in the pure culture (lactate MW2); (B) formate production by *L. lactis* LD61 in the pure culture (formate LD61) and the mixed culture (formate MC) and by *S. aureus* MW2 in the pure culture (formate MW2); (C) acetate production by *L. lactis* LD61 in the pure culture (acetate LD61) and the mixed culture (acetate MC) and by *S. aureus* MW2 in the pure culture (acetate MW2) and ethanol production by *L. lactis* LD61 in the pure culture (EtOH LD61) and the mixed culture (EtOH MC) and by *S. aureus* MW2 in the pure culture (EtOH MW2).

and ethanol were produced. In *L. lactis*, formate is an anaerobic product due to the strong sensitivity of pyruvate formate-lyase to oxygen (29). Similarly, the production of lactate, formate, and acetate was related to anaerobic metabolism in *S. aureus* (17, 45). An anaerobic environment underlies the accumulation of these products. Under our experimental conditions, oxygen was not supplied by aeration in the fermentor. The oxygen concentration was measured at ~80% saturation

at the inoculation time and rapidly dropped to an undetectable level after 4 h (data not shown). Formate production in the mixed culture, earlier and stronger than the cumulative production in pure cultures, revealed a more restricted oxygen condition in the mixed culture. This stronger formate production in the mixed culture was accentuated after glucose exhaustion due to the metabolic change in *S. aureus* toward lactate consumption to produce formate, acetate, and ethanol. The oxygen limitation did not have an impact on *L. lactis* growth, although it seemed to be responsible for the deceleration of *S. aureus* growth.

Transcriptomic design and analysis. To obtain an overall view of the transcriptomic response of *L. lactis* to sharing its environment with *S. aureus*, four kinetic points in the two pure cultures and in the mixed culture were analyzed. They represented exponential, decelerating, early stationary, and late stationary growth phases, i.e., 5, 7.8, 9, and 11.1 h postinoculation, respectively. The array was designed on the basis of the *L. lactis* IL1403 genome sequence (4), was made of PCR fragments spotted onto a nylon membrane, and was employed previously for the analysis of the *L. lactis* LD61 strain (37). The major technical challenge in studying the *L. lactis* transcriptome in mixed cultures was to avoid cross hybridizations with cDNA from the *S. aureus* subpopulation in the mixed culture. Due to the phylogenetic proximity of *L. lactis* and *S. aureus*, about 80% of the array responded positively to the pure population of cDNA from *S. aureus* (data not shown). A previous study analyzing the *L. lactis* transcriptome in mixed cultures with *Saccharomyces cerevisiae* (27) showed that the addition of fragmentized genomic DNA from the microbial partner reduces cross hybridizations. A similar approach was employed in this study by adding *S. aureus* MW2 genomic DNA, which led to a substantial reduction in cross-hybridized signals, as 812 genes (42%, instead of 80%) cross hybridized at least once at the four studied points. Moreover, the cross hybridization was analyzed with *S. aureus* cDNA prepared from RNA in each of the growth kinetics samples, i.e., those from 5, 7.8, 9, and 11.1 h of culture. The vast majority of the 812 genes had constant signals all along the kinetics time course of the *S. aureus* pure culture. Indeed, only 39, 66, and 83 genes in the *S. aureus* pure culture at 7.8, 9, and 11.1 h, respectively, presented a varying signal. As these genes may influence the variations of *L. lactis* gene expression in the mixed culture, they were removed from further analysis. For all the remaining genes, we considered that the signal modifications during the dynamics in the mixed culture were not related to cross hybridizations, as cross hybridizations were constant. Dynamic analyses of pure and mixed cultures were then performed, and genes with significant variations were compared (see Materials and Methods).

Differential gene expression was considered to be specific when expression in the mixed culture (FDR $< 3\%$) but not in the pure culture (FDR $> 3\%$) varied significantly from the reference at a minimum of one point during the course of the culture dynamics. Compared to the levels of expression at 5 h, the expression of 100 genes (33 down- and 67 upregulated) at 7.8 h, 24 genes (12 down- and 12 upregulated) at 9 h, and 25 genes (13 down- and 12 upregulated) at 11.1 h demonstrated specific modifications in the mixed culture. Forty-nine genes with significant expression modifications in both the mixed culture and the pure culture but with opposite expression patterns or differences of more than twofold in expression levels in the mixed and pure cultures were also considered. This analysis led to the identification of 191 genes differentially expressed in the mixed culture at least once during the time course of the culture. In addition, many genes (216 genes) were differentially expressed in the *L. lactis* pure culture, although their expression was unmodified in the mixed culture. This finding is indirect evidence of an opposite expression pattern in *L. lactis* in the mixed culture. Altogether, these results indicated that a large proportion of lactococcal genes (356 genes) showed differential kinetics of expression in pure and mixed cultures (see Table S1 in the supplemental material for a list of all differentially expressed genes). This finding dramatically contrasted with the absence of major changes detected at the macroscopic level in *L. lactis* in the mixed culture and confirmed the interest of the transcriptomic approach for the study of interspecies bacterial interactions.

The effects of *S. aureus* on *L. lactis* were related mainly to nutritional competition. Among the 191 genes displaying differential expression levels in the mixed culture, 48 genes had similar modifications in expression in the *L. lactis* pure culture, but later in growth. This delayed response did not represent a real cellular interaction but rather the earlier evolution of the medium composition in the mixed culture, particularly with respect to the glucose concentration. The changes in the expression of a majority of these genes (25) were likely related to earlier carbon limitation, as these genes overlapped with the previously described carbon starvation stimulon (39). The glucose concentration in the medium of the mixed culture at 7.8 h was only 3 mM, compared to 15 mM in the *L. lactis* pure culture at the same time point (see above). The *L. lactis* glucose limitation threshold was previously determined to be about 15 to 20 mM (39). In the pure culture, *L. lactis* had just entered or approached a state of glucose limitation at 7.8 h, whereas in the mixed culture, it faced clear glucose limitation at the same time point. *L. lactis* responded to this earlier glucose limitation by the induction of alternative sugar uptake and metabolism systems. Some of the corresponding genes (*malQ* and *ypcG*) were overexpressed earlier in the mixed culture than in the pure culture, and some (*dhaM*, *glpF1*, *msmK*, *rbsC*, *rbsK*, and *ypcA*) were expressed in both cultures but with more pronounced overexpression in the mixed culture (Table 2). Components of uncharacterized sugar ABC transporters (encoded by *yngE*, *yngF*, *ypcG*, and *ypcH*) were also upregulated in the mixed culture.

In accordance with the earlier carbon starvation, we observed an advanced decrease in the expression of genes of the growth rate regulon, as about half (20 of 48) of the genes with differential expression belong to this regulon. One of the major cellular responses to growth rate limitation was the underexpression of ribosomal protein-encoding genes (10). As the mixed and pure cultures were in a growth deceleration phase at 9 and 11.1 h, we indeed observed massive underexpression of ribosomal protein-encoding genes (with 19 to 36 of such genes underexpressed). Furthermore, six genes (*rpmB*, *rpmJ*, *rpsJ*, *rpsM*, *rpsO*, and *rpsS*) of the growth rate stimulon were subjected to decreased expression in the mixed culture, suggesting that the *L. lactis* growth rate was reduced to a greater extent than the *S. aureus* growth rate in the mixed culture, although this pattern was not visible at the macroscopic level. These

TABLE 2. Genes discussed in this work that were differentially expressed in pure and mixed cultures over time

Function and gene	Description of gene product[a]	Ratio[b] of expression at 5 h to that at:					
		7.8 h		9 h		11.1 h	
		Pure culture	Mixed culture	Pure culture	Mixed culture	Pure culture	Mixed culture
Amino acid biosynthesis							
serB	Phosphoserine phosphatase		1.76				
Transformation							
comEC	Competence protein ComEC					1.41	
comGA	Competence protein ComGA	1.59				1.89	
comGC	Competence protein ComGC	1.23				1.49	
comGD	Competence protein ComGD					2.03	
Menaquinone biosynthesis							
ispA	Farnesyl diphosphate synthase		1.2	0.6		0.57	
ispB	Heptaprenyl diphosphate synthase component II		1.25				
menB	Dihydroxynaphthonic acid synthase	0.7				0.49	
menD	2-Oxoglutarate decarboxylase	0.55					
menE	*O*-Succinylbenzoic acid-CoA ligase	0.71					
menF	Menaquinone-specific isochorismate synthase	0.44		0.28			
menX	Protein in menaquinone biosynthesis pathway	0.54					
preA	Prenyltransferase		0.76				
Cell envelope							
acmB	*N*-Acetylmuramidase		1.26			0.6	0.72
acmD	*N*-Acetylmuramidase		1.42				0.52
apu	Amylopullulanase	1.43		1.73		1.87	
xynD	Endo-1,4-beta-xylanase D				0.53		0.39
Energy metabolism							
aldB	Alpha-acetolactate decarboxylase		1.48				
mae	Malate oxidoreductase		1.76		0.52		
rbsK	Ribokinase			2.65	15.18		
ypcA	Beta-glucosidase			2.3	2.99		1.85
citB	Aconitate hydratase	1.93	2.47				
citC	Acetate-SH-citrate lyase ligase		1.94				
citD	Citrate lyase acyl carrier protein		2.22 (0.04)		2.02 (0.072)	0.46	
citE	Citrate lyase beta chain		2.1 (0.07)		2.37 (0.029)	0.59	
citF	Citrate lyase alpha chain		1.92 (0.03)		2.19		
citR	Citrate lyase regulator		1.7			0.34	
Adaptation and responses to atypical conditions							
grpE	Chaperone protein GrpE		0.49			0.47	
dnaJ	Chaperone protein DnaJ		0.76				
yahB	Universal stress protein	1.5		2.52	4.72	3.11	7.49
yjaB	Universal stress protein			2.47	3.73		4.8
ymgG	Putative general stress protein 24				4.06		4.41
yobA	Conserved protein			1.94	8.49		7.54
ytaA	Member of universal stress protein family			1.94	3.3	2.47	2.89
ytgH	Putative general stress protein 24			2.17	5.66		9.88
Purine, pyrimidine, nucleoside, and nucleotide metabolism							
carB	Carbamoylphosphate synthetase		0.54		0.25		0.22
dut	Deoxyuridine 5′-triphosphate nucleotide hydrolase				0.63		0.54
pydB	Dihydroorotate dehydrogenase B		0.37				
pyrF	Orotidine-phosphate decarboxylase		0.49		0.51		0.31
pyrR	Pyrimidine operon regulator		0.53				
pdp	Pyrimidine-nucleoside phosphorylase		1.51			0.68	
Regulatory functions							
copR	Transcription regulator				1.82		
fur	Ferric uptake regulator				2.3		
phoU	Phosphate transport system regulator				1.93		
DNA replication and repair							
mutS	DNA mismatch repair protein		1.61	0.64			
mutX	Mutator protein MutT		1.52				
ykjE	MutT/Nudix family protein				0.55		
yqgC	MutT/Nudix family protein				0.59		0.5
Translation							
rpmB	50S ribosomal protein L28			0.68	0.32	0.32	
rpmJ	50S ribosomal protein L36			0.43	0.22	0.33	0.17
rpsJ	30S ribosomal protein S10	0.53		0.46	0.27	0.18	

Continued on following page

TABLE 2—*Continued*

Function and gene	Description of gene product[a]	Ratio[b] of expression at 5 h to that at:					
		7.8 h		9 h		11.1 h	
		Pure culture	Mixed culture	Pure culture	Mixed culture	Pure culture	Mixed culture
rpsM	30S ribosomal protein S13			0.31	0.17	0.22	
rpsO	30S ribosomal protein S15				0.64	0.43	0.2
rpsS	30S ribosomal protein S19					0.21	0.11
argS	Arginyl-tRNA synthetase			0.51	0.4	0.5	0.4
aspS	Aspartyl-tRNA synthetase				0.64	0.29	
glyS	Glycyl-tRNA synthetase alpha chain	0.66		0.64		0.54	
ileS	Isoleucyl-tRNA synthetase				0.59		
leuS	Leucyl-tRNA synthetase		0.63		0.42	0.47	
lysS	Lysyl-tRNA synthetase	0.75		0.33		0.23	
metS	Methyonyl-tRNA synthetase			0.42			
pheT	Phenylalanyl-tRNA synthetase beta chain			0.83		0.57	
serS	Seryl-tRNA synthetase	0.73	2.28		3.64	0.68	
tyrS	Tyrosyl-tRNA synthetase 1				0.46	0.36	
valS	Valyl-tRNA synthetase			0.64	0.46		
Transport							
glpF1	Glycerol uptake facilitator			8.33	15.47	7.01	10.14
msmK	Multiple-sugar ABC transporter ATP binding protein			2.84	7.86	1.92	3.3
rbsC	Ribose ABC transporter permease protein			1.63	6.36		1.55
yngE	Sugar ABC transporter ATP binding protein		1.58			0.28	
yngF	Sugar ABC transporter permease protein		1.74			0.34	
ypcG	Sugar ABC transporter substrate binding protein		1.8	2.78	7.01		2.61
ypcH	Sugar ABC transporter permease protein			2.33	5.19		
mtsA	Manganese ABC transporter substrate binding protein		2.54			0.25	
mtsB	Manganese ABC transporter ATP binding protein		2				
mtsC	Manganese ABC transporter permease protein		2.43				
ykjB	Putative manganese transporter		1.67				
Unknown function							
yihF	Putative glycerate kinase				2.44		2.45
ymgH	Unknown protein				3.43		3.8
ymgI	Unknown protein				3.66	2.81	3.98
ymgJ	Unknown protein			2.25	3.42	3.24	4.73
ytgA	Unknown protein				5.75		9.16
ytgB	Unknown protein		1.98		4.39		7.87

[a] CoA, coenzyme A.
[b] *P* values above the threshold are shown in parentheses.

genes may be used as accurate sensors to detect slight reductions in the growth rate of *L. lactis*.

Ten genes encoding tRNA synthetases (*argS*, *aspS*, *glyS*, *ileS*, *leuS*, *lysS*, *metS*, *pheT*, *tyrS*, and *valS*) were underexpressed from 7.8 h in both pure and mixed cultures. Only the *serS* tRNA synthetase gene was overexpressed in the mixed culture at 7.8 h, and this upregulation was maintained at 9 h. All amino acids in the culture media were quantified. All but serine and threonine remained present at 25 h and were similarly consumed in the three types of cultures (data not shown). In the mixed culture, serine and threonine were exhausted at 8 h. This exhaustion resulted from the specific consumption by *S. aureus*, as these amino acids were similarly consumed in the *S. aureus* pure culture but only weakly consumed in the *L. lactis* pure culture (data not shown). Transcriptomic responses in serine metabolism, but not in threonine metabolism, were observed. In parallel to serine exhaustion, *serB*, encoding phosphoserine phosphatase, and *yihF*, encoding a putative glycerate kinase acting upstream of the serine biosynthesis pathway, were specifically overexpressed in the mixed culture. Thus, *L. lactis* responded specifically to nutritional modifications of the environment induced by *S. aureus*. While the decrease of expression of most aminoacyl-tRNA synthetase genes is related to the slowing down and arrest of growth (8, 13), the specific induction of *serS* was probably related to serine exhaustion.

Genes from the de novo pyrimidine biosynthetic pathway (*carB*, *dut*, *pydB*, *pyrF*, *pyrZ*, and *pyrR*) were specifically underexpressed in the mixed culture. In this pathway, only *pdp*, encoding a pyrimidine-nucleoside phosphorylase involved in cytosine-to-cytidine conversion, was specifically overexpressed in the mixed culture. In *L. lactis* grown in mixed cultures with *S. cerevisiae*, similar *pyr* gene downregulation due to the ethanol produced by the yeast was observed (27). One cannot exclude the possibility that the *pyr* gene underexpression observed in the mixed culture may be related to the ethanol produced by *S. aureus*.

Several regulators involved in copper, ferric, and phosphate transport (*copR*, *fur*, and *phoU*) were overexpressed in the mixed culture, but without clear repercussions on the expression of genes controlled by these regulators. A specific response in manganese transport was observed. Although the CDM used did not contain a specific manganese source, trace amounts were likely supplied by other chemical components. Manganese participates as a cofactor in several vital metabolic processes (7, 9). It is thus likely that *L. lactis* and *S. aureus* compete for the Mn^{2+} amounts present in the medium. The

mtsABC genes, encoding manganese transport system components, were specifically overexpressed (by more than twofold) in the mixed culture at 7.8 h. Lactococcal *mtsABC* displays homologies to *Bacillus subtilis mntABD* and *Lactobacillus plantarum mtsCBA*, whose expression is strongly increased under conditions of manganese starvation (20). A second manganese transport system involving MntH in *B. subtilis* (36) and MntH1 and MntH2 in *L. plantarum* (20) has been described. These three proteins show homologies to the putative manganese transporter encoded by *ykjB*, which was also specifically overexpressed in the mixed culture at 7.8 h. The specific overexpression of genes involved in manganese uptake suggests that amounts of manganese present in the medium were sufficient for the *L. lactis* pure culture but led to competition for this cofactor in the mixed culture. Cellular functions in *L. lactis* and *S. aureus* that may be affected by the reduction of the manganese concentration in the mixed culture remain unidentified.

***S. aureus* did not induce a major stress response in *L. lactis*.** Under different stress conditions, *L. lactis* is able to activate the synthesis of general stress response proteins such as chaperones and proteases (15, 16, 23, 35). Only *grpE* and *dnaJ* were specifically underexpressed in the mixed culture at 9 h, suggesting that the presence of *S. aureus* did not represent a major stress stimulus for *L. lactis*. However, some putative universal stress protein-encoding genes (*yahB*, *yjaB*, *ymgG*, *yobA*, *ytaA*, and *ytgH*) with uncharacterized functions were specifically overexpressed or overexpressed to a greater extent in the mixed culture in stationary phase (9 and 11.1 h). Among them, both *ymgG* and *ytgH* show homologies to the *Enterococcus faecalis gls24* gene, predicted to code for a general stress protein involved in survival and virulence and induced by different starvation conditions as well as chemical stresses (18, 43). *ytgH*, but not *ymgG*, was reported previously to respond to various environmental stress conditions (19). These genes form transcriptional units with upstream genes *ymgHIJ* and *ytgAB*, which were also all specifically overexpressed or expressed to a greater extent in the mixed culture. The biological functions of the *gls24* family members remain unclear (19). Further analyses are required to determine whether the overexpression of those two operons was related to carbon starvation or to a stimulating molecule produced by *S. aureus* in the environment. Finally, some genes associated with DNA repair (*mutS* and *mutX*) were overexpressed, although *ykjE* and *yqgC*, two putative members of the Nudix family involved in the degradation of potentially mutagenic X-linked nucleotide diphosphates (3, 28), were underexpressed.

Taken together, these results clearly showed that the presence of *S. aureus* in the medium does not generate major stress for *L. lactis*. This outcome was expected, as most characterized stress-associated genes are generally regulated by massive environmental (temperature and pH, etc.) or medium composition modifications. In our model, the main stress-generating parameters were controlled, but minor and unidentified stresses may be triggered by *S. aureus*, directly or indirectly. The putative stress protein-encoding genes identified may then be involved in a specific response to interaction between the two microorganisms. Identifying their biological functions will facilitate the characterization of this interaction.

We have demonstrated that the oxygen level in the environment was rapidly reduced, as shown by the quantification of metabolites released into the medium. The overall response of *L. lactis* to oxygen limitation was not detected at the transcriptomic level. Genes whose expression is modified in the presence of oxygen have recently been described (32). Under our conditions, 82% of these oxygen-regulated genes had unmodified expression in pure and mixed cultures at 7.8 h. Furthermore, equal repartition of over- and underexpression was observed for the 18% of genes with varying expression. More particularly, genes directly involved in oxygen metabolism (*pdhABCD*, *noxE*, *pfl*, *ahpCF*, and *sodA*) had unmodified expression in the mixed culture compared to that in the *L. lactis* pure culture. Taken together, these results indicated that cells at the reference point (5 h) were already in a state of oxygen limitation or that, if further modifications existed, they were too minor to trigger significant transcriptomic rearrangements. Unexpectedly, the expression of genes associated with electron transfer in respiration chains in *L. lactis* was specifically modified in the mixed culture compared to that in the pure culture. The *ispA* and *ispB* genes, encoding isoprenyl diphosphate synthases involved in menaquinone side chain elongation (25), were specifically overexpressed in the mixed culture at 7.8 h, although the prenyltransferase gene *preA* was concomitantly underexpressed. In addition, *cydA* and many genes involved in menaquinone biosynthesis (*menBDEFX*) were specifically underexpressed in the pure culture at 7.8 h compared to expression at 5 h, although their expression was maintained in the mixed culture, suggesting that the menaquinone biosynthetic pathway was sustained in the mixed culture. An example of cellular communication between *L. lactis* and group B streptococci through menaquinone exchange, in which *L. lactis* can cross-feed menaquinones to streptococci through cell lysis or direct cell-cell interaction, has been described recently (40). A similar situation may occur with *S. aureus.* The signals and biological relevance of this interaction remain to be analyzed.

For intraspecies communication mechanisms, competency is controlled in many gram-positive bacteria by a quorum-sensing system activated by a competence-stimulating peptide (33, 42). *L. lactis* has never been identified as being able to develop a natural competence pathway, but genes required for late competence steps, such as DNA entry pore formation, are present (4). Under our conditions, the overexpression of those competence-related genes (*comEC*, *comGA*, *comGC*, and *comGD*) was observed in the pure culture, although not in the mixed culture. This specific downregulation of competence genes in the mixed culture remains unexplained and may be due to interference triggered by *S. aureus*.

Modulation of *L. lactis* genes associated with technological properties. Citrate is present in many natural media, including milk, and can be used by lactococci as a carbon source. Citrate is converted to pyruvate, yielding the formation of acetolactate, acetoin, and diacetyl, components with flavor properties essential for the quality of some dairy products (12). *L. lactis* LD61 possesses the plasmidic citrate permease-encoding gene *citP* for efficient citrate uptake (11, 37). The *citCDEF* operon involved in citrate utilization, *citB*, and the citrate lyase regulator *citR* were globally overexpressed, with about twofold induction, in the mixed culture at 7.8 and 9 h. Genes encoding enzymes localized downstream of the pyruvate pool, such as malic enzyme (*mae*) and one enzyme involved in the diacetyl/acetoin pathway (*aldB*), were specifically overexpressed at

7.8 h. This finding suggests that the citrate pathway was activated at the transcriptomic level, directly or indirectly, by the presence of *S. aureus* in our model. Such interaction may have an impact on flavor compound formation in the food matrix.

The lysis of *L. lactis* in the mixed culture was more pronounced than that in the pure culture whatever the time point, as suggested by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of culture supernatants (data not shown). This pattern was not linked to more pronounced overexpression of phage-associated genes in the mixed culture than in the pure culture. Among the 40 phage-related genes overexpressed in the pure culture, 20 had unmodified expression in the mixed culture. However, we cannot exclude the possibility that a phage repertoire different from that in *L. lactis* IL1403 is present in *L. lactis* LD61, which would in turn also contribute to the more pronounced lysis observed for this strain in mixed culture. The differential cell lysis in the mixed culture may also be related to the increased sensitivity in the mixed culture of *L. lactis* peptidoglycan, a major component of the gram-positive bacterial cell wall that ensures cell rigidity and stability. None of the genes involved in peptidoglycan biosynthesis were differentially expressed. Bacteria produce peptidoglycan hydrolases with large repertoires of cellular functions. Among the five peptidoglycan hydrolases in *L. lactis* identified so far (5, 21, 38), only two minor hydrolases, those encoded by *acmB* and *acmD*, were specifically overexpressed at 7.8 h in the mixed culture. The gene *xynD* (renamed *pgdA*), encoding a peptidoglycan *N*-acetylglucosamine deacetylase whose activity increases resistance to autolysis or exogenous muramidases (30), was underexpressed in the mixed culture. Inversely, *apu*, involved in the degradation of polysaccharide (30), was specifically overexpressed in the pure culture. The combined differential expression profiles of genes involved in peptidoglycan physiology may participate to some extent in the moderately increased cell lysis observed in the mixed culture.

Concluding remarks. Our study demonstrated that *L. lactis* growth was hardly affected by the presence of *S. aureus* at the macroscopic level. However, the transcriptomic analysis detected a large set of genes that were differentially expressed, confirming the interest of such a global approach for the study of interspecies bacterial interactions. Several nutritional or trophic interactions were revealed or at least suggested by the transcriptomic analysis. Although the main transcriptomic repercussion in the mixed culture was the earlier evolution of the environmental composition, some responses likely reflect more specific phenomena of interaction between *L. lactis* and *S. aureus*. It has yet to be determined whether these modifications correspond to direct or indirect bacterial interaction and whether they are actively, or not, inflicted by *S. aureus*. Moreover, it remains to be seen if these modifications are specific to *S. aureus* or more generally linked to any bacterial partner. On the molecular scale, remarkable effects of *L. lactis* on *S. aureus* were demonstrated, with *L. lactis* dramatically influencing the expression patterns of various virulence-associated genes, as described elsewhere (14).

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